JOURNAL OF APPLIED ENTOMOLOGY

J. Appl. Entomol.

ORIGINAL CONTRIBUTION

Suppression of aphids by augmentative release of larvae of flightless *Harmonia axyridis*

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Keywords
augmentative biological control, genetic improvement, larval release of flightless ladybirds

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Received: April 3, 2013; accepted: September 4, 2013

doi: 10.1111/jen.12090

Abstract

The release of the larvae of flightless ladybird beetles could extend the duration of effective control because of the longer presence of adult stages, and the per individual production cost for juvenile stages is lower than that for adult stages. A preliminary release experiment was conducted to assess the effectiveness in controlling two aphid species, *Aphis gossypii* and *Aulacorthum solani*, using second instars of a flightless strain of *Harmonia axyridis*. The number of *A. gossypii* was suppressed in greenhouses that contained the flightless strain compared with greenhouses that contained the wild-type strain. In one of two replicates, more flightless adults were observed on the plants for longer than wild-type adults. However, it was not clear whether the extension of the residence period contributed to the effectiveness in controlling aphids directly, because the population of ladybirds in the greenhouses consisted of both larvae and adults during parts of the experimental period. In flightless *H. axyridis*, the release of larvae was more effective in suppressing *A. solani* than the release of adults. These results suggest that it may be more effective to release larvae of flightless *H. axyridis* than wild-type larvae or flightless adults.

Introduction

Ladybird beetles are widely used to control aphids through augmentation by translocation or mass rearing and release (Dixon 2000). Previous studies suggested that only larval stages would provide control, because adults tended to disperse from plants (Hagen 1962; Marples et al. 1993; Dreistadt and Flint 1996; Ferran et al. 1996; Trouve et al. 1997; Dixon 2000). However, flightless strains have been established for *Harmonia axyridis* Pallas (Ferran et al. 1998; Tourniaire et al. 2000; Seko et al. 2008; Nakayama et al. 2010) and *Adalia bipunctata* Linnaeus (Marples et al. 1993; Ueno et al. 2004; Lommen et al. 2008, 2013). Adults of these flightless strains have been shown to be effective against aphids, such as *Phorodon humuli* Schrank (Weissenberger et al. 1999), *Myzus persicae* Sulzer (Lommen et al. 2008; Adachi-Hagimori et al. 2011) and *Aphis gossypii* Glover (Seko et al. 2008; Kunimoto 2010; Iguchi et al. 2012).

Even though increasing the effectiveness of ladybirds through selective breeding for reduced flight ability improves the cost–effectiveness, their use continues to be costly (Dixon 2000; Sighinolfi et al. 2008). Adachi-Hagimori et al. (2011) suggested that releasing the larvae of a flightless strain could extend the duration of effective control because the adult stages would be present over a longer period, and it is possible to reduce the cost of biological treatments with *H. axyridis* by releasing young larvae of the flightless strain because mass rearing to the more voracious fourth-instar larvae and adults would be unnecessary (Seko et al. 2008). For example, it has been estimated that the production cost of one adult flightless *H. axyridis* is equal to that of 10 s instars (Adachi-Hagimori et al. 2011; Seko 2011). If releasing the larvae is as effective

as or more effective than releasing adults, then use of the larvae would be more cost–effective. However, their effectiveness in controlling aphids and the potential economic benefits compared with the use of adults have not been studied. Kunimoto (2010) and Adachi-Hagimori et al. (2011) demonstrated that the larvae of flightless *H. axyridis* are effective against *Lipaphis erysimi* Kaltenbach on non-heading *Brassica* cultivars and against *A. gossypii* on chrysanthemum plants, respectively. However, in their studies, the study period was only about 3 weeks, suggesting a need for long-term experiments to observe the dynamics of aphid populations and the flightless strain of ladybirds after emergence. Although Lommen et al. (2013) suggested that releasing larvae of wingless *A. bipunctata* can reduce the impact of honeydew from *Eucallipterus tiliae* (L.) outbreaks on urban lime trees, it is difficult to evaluate their effectiveness in controlling aphids and the potential economic benefits because adults of wingless *A. bipunctata* were also released on the same trees from day 7 after the first release of larvae onwards (Lommen et al. 2013).

The goal of our research is to answer three questions with regard to the use of *H. axyridis* larvae of the flightless strain as a biological control: (i) 'Is the release of larvae of the flightless strain more effective than that of larvae of the wild-type (non-selected) strain, because of the presence of adult stages over a longer period?' (ii) 'Is the release stage of flightless *H. axyridis* important in influencing its effectiveness in controlling aphids?' (iii) 'Are there potential economic benefits of releasing larvae?' A preliminary release experiment was conducted to assess the effectiveness in controlling two aphid species, *A. gossypii* and *Aulacorthum solani* Kaltenbach, using second instars of a flightless strain of *H. axyridis*. There are three types of release methods for *H. axyridis*: egg masses, larvae and adults (Tedders and Schaefer 1994; Ferran et al. 1996; Trouve et al. 1997; Kitagami and Ohkubo 1998; Kuroda and Miura 2003). Although the use of egg masses and first instars of ladybirds would be less expensive, they are not likely to be effective because the larvae have a low dispersal capacity (Dixon 1959, 1970; Kawai 1978; Lee and Kang 2004; Seko and Miura 2008). Therefore, second instars were used for release in our study.

Previous studies suggest that depression of viability during immature stages and fecundity in a flightless strain of *H. axyridis* affects the effectiveness of aphid control through the higher mortality of ladybirds released and the low incidence of their offspring (Seko and Miura 2009; Seko et al. 2012). Therefore, the effectiveness for control of aphids could be influenced not only by the flight ability, but also by the quality of fitness components. In this study, we also compared flight ability and fitness parameters, that is, hatching rate, developmental period, proportion emergence and early fecundity between flightless and wild-type strains of *H. axyridis* under laboratory conditions.

Materials and Methods

Ladybirds

Adults and second instars of the flightless strain of *H. axyridis* were provided from the plant culture of Agriseect (Tokyo, Japan). This strain was originally established by artificial selection for reduced flight ability at the NARO Western Region Agricultural Research Center (Nakayama et al. 2010). The selection protocol is described in detail in Nakayama et al. (2010). Briefly, the flight distance of males and females (48 each) was measured using the flight mill system (Seko et al. 2008); the males and females (15 each) that covered the shortest distances were selected and allowed to mate to propagate the next generation. This procedure was carried out for more than 30 generations. The flightless adults have normal elytra and wings, but they cannot flap their wings.

Another strain with randomly selected offspring, thereby imposing no artificial selection on flight ability (i.e. wild-type strain), was established using the method of Seko et al. (2008) and Nakayama et al. (2010). One hundred and fifty adults of *H. axyridis* were collected in Fukuyama (Western Japan, 34°28′ N, 133°23′E) during late April 2010 and were allowed to mate and lay eggs on tissue paper at a density of 50 individuals per rectangular plastic container (16 × 22 × 8 cm). Thirty egg clusters of *H. axyridis* were collected, and 130 hatchlings were randomly selected from these egg clusters (first generation). Larvae were reared at a density of 20 individuals per circular plastic container (diameter 10 cm, height 5.5 cm). After emergence, 60 adults (30 males and 30 females) were selected randomly and mated in a square plastic container. The same procedure as described above was repeated for all subsequent generations. In release experiments, second instars of the wild-type strain were used in the second–third and ninth–tenth generations, respectively.

Development, reproductive traits and flight performance of each strain

Several parameters of development and reproduction were measured for the flightless and the wild-type

strains using the method of Seko et al. (2012). The number of hatchlings per 12 h was counted for 2 days after the first hatchling was confirmed. A random sample of 80 and 90 hatchlings in the flightless and wild-type strains, respectively, was individually introduced into a plastic cup (diameter 4.5, height 3.5 cm) and fed cucumber leaves with *A. gossypii*. The developmental period (from hatching to adult emergence) was monitored daily, and the proportion emergence was noted. Pairs of one male and one female were randomly established on the day following adult emergence. Early fecundity was measured daily for the first 15 days after the onset of oviposition. The flight distances for 1 h of 27–42 adults of each strain were measured using the flight mill system (Seko et al. 2008). The flight distance of the wild-type strain was measured in the second generation. These experiments were conducted under a 16L : 8D photoperiod at 25°C and relative humidity of 40–60%.

Release experiments

First, augmentative release of *H. axyridis* second instars was conducted to compare suppression of *A. gossypii* and the residence time of *H. axyridis* on eggplants between greenhouses in which larvae of flightless and wild-type strains were released (Trials 1 and 2). Second, the numbers of *A. solani* and *H. axyridis* were compared between greenhouses in which adults and second instars of the flightless strain were released to evaluate the effectiveness of second instars in controlling *A. solani* (Trial 3). Table 1 shows the overview of conditions during each release experiment in greenhouses with cultivated eggplants.

In Trial 1, the release experiment was carried out at the NARO Western Region Agricultural Research Center, Japan, from 19 April to 23 July 2010. The plots were set up in three small-scale greenhouses with cultivated eggplants, *Solanum melongena* (L.). Each of the three plots consisted of two rows of plants, with 120 cm between rows and 50 cm between plants, so that each plot contained 30 plants. Eggplant seedlings with five leaves were transplanted on 14 April. After the door of greenhouses had been opened for 4–5 days to attract *A. gossypii* to greenhouses, it had been closed not to let wild-type *H. axyridis* that were left from the treatment in which the wild-type strain was released (wild-type plot) and/or indigenous ladybirds in greenhouses. The incidence of *A. gossypii* was observed in all treatments on 19 April (on day 1). The initial densities of *A. gossypii* per eggplant were 2.0, 7.0 and 1.0 individuals in the treatment in which the flightless strain was released (flightless plot), the wild-type plot and the no-release plot, respectively. Second instars of each strain were released on eggplant plants with *A. gossypii* on 29 April and 5, 13 and 24 May. The release density was 10 s instars per eggplant. The numbers of *A. gossypii* on 20 selected plants in each plot were counted at intervals of 3 or 4 days. Six leaves were randomly selected per eggplant to count the total aphid numbers. Fourth instar and adult *H. axyridis* on all plants and on the ceilings of the greenhouses were also counted. Trial 2, which involved a release experiment similar to Trial 1, was carried out from 21 April to 14 July 2011. Eggplant seedlings with five leaves were transplanted on 20 April 2011. The incidence of *A. gossypii* was observed in all treatments on 28 April (on day 8). The initial

Table 1 Overview of conditions during each release experiment in greenhouses with cultivated eggplants

Objectives	Trial	Experimental period	Aphid species	Strain of *Harmonia axyridis*	Numbers of ladybirds released (Numbers of times released)
Comparison of suppression of an aphid species and the incidence of *H. axyridis* between greenhouses in which second instars of flightless and wild-type strains were released	1	19 April–23 July 2010	*Aphis gossypii*	Flightless (larvae)	300 (4)
				Wild-type (larvae)	300 (4)
				No-release	0 (0)
	2	21 April–14 July 2011	*Aphis gossypii*	Flightless (larvae)	300 (5)
				Wild-type (larvae)	300 (5)
				No-release	0 (0)
Comparison of suppression of an aphid species and the incidence of *H. axyridis* between greenhouses in which adults and second instars of the flightless strain were released	3	17 November 2009–12 January 2010	*Aulacorthum solani*	Flightless (larvae)	600 (3)
				Flightless (adults)	120 (3)
				No-release	0 (0)

densities of *A. gossypii* per eggplant were 23.5, 11.0 and 5.5 individuals in the flightless, the wild-type and the no-release plots, respectively. Second instars of each strain were released on eggplants with *A. gossypii* on 2, 9, 17 and 30 May and 6 June.

The release experiment in Trial 3 was carried out at the Agriculture Research Institute, Tokushima Agriculture, Forestry and Fisheries Technology Support Center, Japan, from 17 November 2009 to 12 January 2010. The plots were set up in three small-scale greenhouses with cultivated eggplants. Each of the three plots consisted of three rows of plants, with 120 cm between rows and 90 cm between plants, so that each plot contained 60 plants. Eggplant seedlings were transplanted on 30 September 2009. *Aulacorthum solani* raised on broad bean seedlings, *Phaseolus vulgaris* L., were released on eggplants on 13, 20 and 26 October and 4 November. The initial densities of *A. solani* per eggplant just before the release experiment were 55.5, 47.5 and 3.0 individuals in the treatment in which *H. axyridis* second instars were released [flightless (larvae)], the treatment in which *H. axyridis* adults were released [flightless (adults)] and the no-release plot, respectively (on day 1). Second instars and adults of flightless *H. axyridis* were released on eggplants with *A. solani* on 17, 24 and 30 November. The release density was 10 s instars and two adults per eggplant. The numbers of *A. solani*, larvae and adults of *H. axyridis* on 30 selected plants in each plot were counted at intervals of 1 week. Ten leaves were randomly selected per eggplant to count the total numbers of aphids and of *H. axyridis* larvae and adults.

Statistical analyses

The normality of the data was assessed using the Shapiro–Wilk test. Normally distributed variables for hatching rate (after arcsine transformation) and early fecundity were compared between strains using Student's *t*-test. Non-normally distributed data for the developmental period and the flight distance were compared using the Wilcoxon rank sum test. For proportional emergence, the chi-square test was used. These statistical analyses were performed using JMP, version 9 (SAS Institute Japan, Tokyo, Japan). Analyses by the generalized linear mixed model (GLMM) with a Poisson error distribution (link = log) were conducted using R version 2.14.1 (R Development Core Team 2011) with the lme4 package (version 0.999375-42) to test for the effects of the treatment on *A. gossypii*, *A. solani* and *H. axyridis* abundance. The independent variables were treatment, day and the interaction as a fixed effect and eggplant plants as a random effect. Treatment and plant were the categorical variables, and day was the continuous variable. The numbers of *H. axyridis* on the ceilings of the greenhouses were analysed by the generalized linear model (GLM) with the glm.nb function (R version 2.14.1, Mass package, version 7.3-16) because of overdispersion. The independent variable was treatment and day as a fixed effect. The total number of *H. axyridis* in each greenhouse was treated as an offset variable. These best-fitting models were selected based on the Akaike information criterion.

Results

Development, reproductive traits and flight performance of each strain

Table 2 shows the differences in hatching rate, proportion emergence, early fecundity, developmental period and flight distance between the strains of *H. axyridis* used in this study. The hatching rate and the proportion emergence of the flightless strain were significantly lower than those of the wild-type strain. Early fecundity did not differ between the strains. The developmental period of the flightless strain was longer than that of the wild-type strain in both sexes. The flight distance of the flightless strain was significantly shorter than that of the wild-type strain.

Release experiments

Trials 1 and 2

Figure 1 shows the numbers of (a) *A. gossypii*, (b) fourth-instar *H. axyridis*, (c) adult *H. axyridis* on eggplants and (d) adult *H. axyridis* on the greenhouse ceiling. In Trial 1, the number of *A. gossypii* reached about 1600 individuals on six leaves per plant on day 33 after the investigation start date in the no-release plot (fig. 1a). Thereafter, the number of *A. gossypii* in the no-release plot decreased because the outbreak of aphids caused severe damage to many leaves of the eggplant plants. The incidence of *A. gossypii* was suppressed in the flightless plot compared with the no-release plot and the wild-type plot (fig. 1a). With regard to the number of *H. axyridis* fourth instars, many larvae were observed in the flightless plot from days 50 to 64 (fig. 1b). The total number of fourth instars reached 390 individuals in the flightless plot on day 57. With regard to the number of *H. axyridis* adults on eggplants, the adults were observed to be present for a longer time in the flightless plot than in

Table 2 Hatching rate, proportion emergence, early fecundity, developmental period and flight distance of flightless and wild-type strains of *Harmonia axyridis*

Strain	n[a]	Hatching rate [median (min.–max.)][b]	n	Proportion emergence[c]	Flight distance for 1 h [median (min.–max.), in m][d] n	Male	n	Female
Flightless	49	0.70 (0–1)	90	0.64	42	0 (0–1138.6)	42	0 (0–7.2)
Wild-type	42	1.0 (0.4–1)***	80	0.81*	27	1151.1 (65.3–1858.7)***	27	839.8 (78.8–1913.8)***

Strain	Developmental period [median (min.–max.)][d] n	Male	n	Female	n	Early fecundity[e] (mean ± SD)
Flightless	33	14.0 (12.0–18.0)	25	14.0 (13.0–15.0)	19	190.6 ± 161.9
Wild-type	26	13.0 (13.0–17.0)**	39	13.0 (12.0–15.0)**	23	270.6 ± 159.2 ns

[a]Number of egg clusters.
[b]Student's *t*-test was conducted after arcsine transformation.
[c]Chi-square test was conducted.
[d]Wilcoxon rank sum test was conducted.
[e]Student's *t*-test was conducted. Early fecundity (expressed as no. eggs per female) was measured daily for the first 15 days after the onset of oviposition.
*P < 0.05; **P < 0.01; ***P < 0.0001; ns, not significant.

the wild-type plot (fig. 1c). The number of adults on the ceiling of the greenhouse was lower in the flightless plot than in the wild-type plot (fig. 1d).

In Trial 2, the number of *A. gossypii* reached about 3000 individuals on six leaves per plant on day 36 in the no-release plot (fig. 1a′). The incidence of *A. gossypii* was suppressed in the flightless plot compared with the no-release plot, as well as with the flightless plot in Trial 1 (fig. 1a′). The number of *A. gossypii* was lower in the flightless plot than in the wild-type plot from days 61 to 82 (fig. 1a′). More fourth-instar *H. axyridis* were observed in the flightless plot than in the wild-type plot (fig. 1b′). The incidence of flightless adults on eggplants was about the same as for the wild type (fig. 1c′). There were few adults on the greenhouse ceiling in the flightless plot (fig. 1d′).

Table 3 summarizes the results of the corresponding GLMMs. There were significant treatment × day interactions (no release and wild type) on the number of *A. gossypii* and *H. axyridis* larvae and adults in both Trials 1 and 2. There were significant treatment effects (no release and wild type) on the number of *A. gossypii* and *H. axyridis* adults in both Trials 1 and 2. Although there was no treatment effect on the number of *H. axyridis* fourth instars in Trial 1, the GLMMs revealed a significant treatment effect in Trial 2. In the numbers of *H. axyridis* on the ceilings, the GLM revealed a significant treatment × day interaction effect (coefficient = 0.05, $z = 3.09$, $P < 0.01$), but no treatment effect (coefficient = −0.303, $z = -0.294$, $P > 0.05$) or day effect (coefficient = −0.011, $z = -0.91$, $P > 0.05$) in Trial 1. The GLM revealed a significant day effect (coefficient = 0.07, $z = 3.39$, $P < 0.01$), but no treatment effect (coefficient = −3.636, $z = -0.45$, $P > 0.05$) or treatment × day interaction effect (coefficient = 0.0007, $z = 0.006$, $P > 0.05$) in Trial 2.

Trial 3

Figure 2 shows the numbers of (a) *A. solani*, (b) larvae of *H. axyridis* and (c) adult *H. axyridis*. The number of *A. solani* reached about 850 individuals on ten leaves per plant on day 57 in the no-release plot (fig. 2a). The incidence of *A. solani* was suppressed in the plot in which second instars were released compared with the no-release plot (fig. 2a). From day 35, the number of *A. solani* was lower in the plot in which *H. axyridis* second instars were released than in the plot in which *H. axyridis* adults were released (fig. 2a). The number of *H. axyridis* adults increased with the reduction in larvae in the plot in which second instars were released and vice versa in the plot in which adults were released (fig. 2b,c).

Table 4 summarizes the results of the corresponding GLMMs. In the number of *A. solani*, there was a significant treatment × day interaction effect (no release), but no treatment effect (no release). The GLMMs revealed both significant treatment effect (adults) and treatment × day interaction effect (adults). The GLMMs revealed significant treatment effects (adults) and treatment × day interaction effects (adults) on the number of *H. axyridis* larvae and adults.

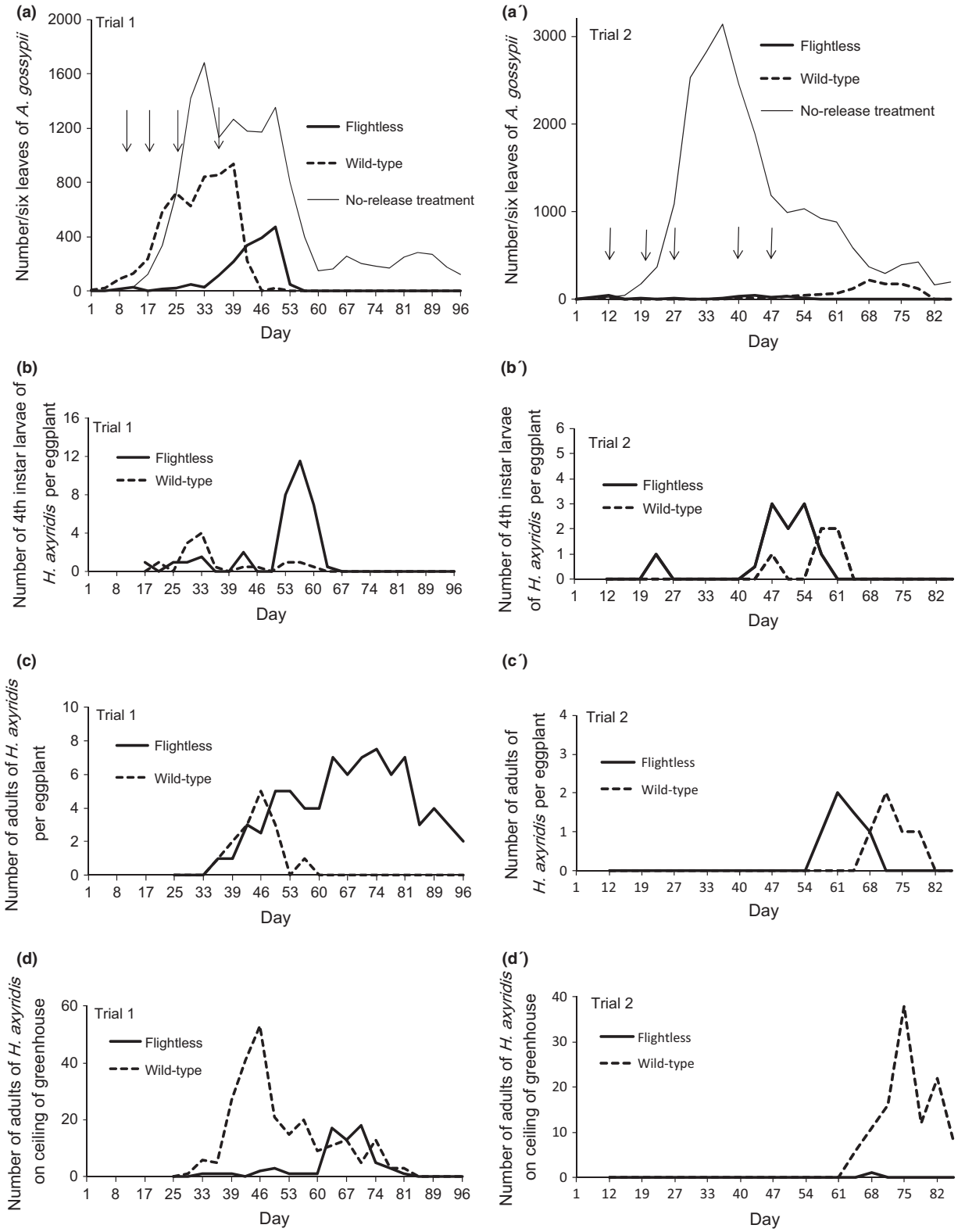

Fig. 1 Variation in time of the number of (a) and (a′) *Aphis gossypii* on six leaves per plant, (b) and (b′) fourth instar *Harmonia axyridis* per plant, (c) and (c′) adult *H. axyridis* per plant and (d) and (d′) adult *H. axyridis* on the ceilings of small-scale greenhouses. All values are expressed as the median with the exception of (d) and (d′). Arrows show the day on which second-instar *H. axyridis* were released in each plot.

Table 3 Results for generalized linear mixed models examining the effects of variables on the numbers of *Aphis gossypii* and *Harmonia axyridis* (fourth instars and adults)

		Trial 1		Trial 2	
Species	Factor	Coefficient	Wald statistic	Coefficient	Wald statistic
A. gossypii	Treatment (flightless)	0		0	
	Treatment (wild type)	1.226	12.29***	−1.361	−12.47***
	Treatment (no release)	1.270	12.73***	3.487	32.48***
	Day	−0.011	−76.94***	−0.013	−29.81***
	Treatment × Day (flightless)	0	0	0	0
	Treatment × Day (wild type)	−0.009	−54.53***	0.047	90.02***
	Treatment × Day (no release)	0.008	46.84***	0.009	20.44***
Larvae of *H. axyridis*	Treatment (flightless)	0		0	
	Treatment (wild-type)	0.231	1.50 ns	−1.054	−4.81***
	Day	−0.0005	−0.54 ns	0.001	0.78 ns
	Treatment × Day (flightless)	0		0	
	Treatment × Day (wild type)	−0.018	−12.15***	0.020	7.07***
Adults of *H. axyridis*	Treatment (flightless)	0		0	
	Treatment (wild type)	0.439	3.41***	−1.710	−4.36***
	Day	0.024	30.02***	0.037	11.09***
	Treatment × Day (flightless)	0		0	
	Treatment × Day (wild type)	−0.031	−20.37***	0.027	5.21***

The listed variables were selected as the minimum Akaike information criterion model for each trial.
***$P < 0.0001$; ns, not significant.

Discussion

In Trials 1 and 2 of our study, even though the two trials varied in their results in the population dynamics of *A. gossypii* and *H. axyridis* on eggplants because it might be partially the results of the different timing of these replicates, both trials showed that the number of *A. gossypii* was suppressed in plots that contained the flightless strain compared with plots that contained the wild-type strain (fig. 1). The GLMMs revealed significant treatment and treatment × day interaction effects on the number of *A. gossypii* and *H. axyridis* adults (table 3), suggesting that the incidence of these species varied between the flightless and the wild-type plots. In one of two replicates, more flightless adults were observed on the plants for longer than wild-type adults. However, it was not clear whether the longer residence period of flightless adults on eggplant supplied effectiveness in controlling aphids directly, because the population of ladybirds in the greenhouses consisted of both larvae and adults during parts of the experimental period. The release of second instars was more effective in suppressing *A. solani* than that of adults, suggesting that the release stage of flightless *H. axyridis* is an important factor influencing its effectiveness in controlling *A. solani*.

The appetitive flight of ladybirds is induced by the ensuing shortage of prey (Hodek et al. 1993). The movement patterns of *H. axyridis* adults are possibly determined by the distribution patterns of resources in a habitat (Osawa 2000). In our study, more *H. axyridis* adults were observed on the greenhouse ceiling in the wild-type plot than in the flightless plot (fig. 1d,d′), suggesting that the wild-type adults could fly away. Some adults were observed on the ceilings in the flightless plot. Although we cannot exclude the possibility of entering wild-type *H. axyridis* in greenhouses because there was a slight gap between doors in the opening of greenhouses, this result corresponds to the presence of adults of the flightless strain with some flight ability (table 2). In Trial 1, more *H. axyridis* adults were observed on the plants for longer in the flightless plot than in the wild-type plot (fig. 1c). On the other hand, the incidence of flightless adults on eggplants was on the same level as the wild types in Trial 2 (fig. 1c′), possibly because there were fewer *A. gossypii* in the flightless plot than in the wild-type plot after emergence of *H. axyridis*.

In Trial 1, GLMMs revealed a significant treatment × day interaction effect, but no treatment effect on the number of fourth instars of *H. axyridis* (table 3). This might be because the timing of the incidence varied between the flightless and the wild-type plots, but no difference between them was found with regard to the total amount of fourth instars throughout the experimental period. In our laboratory experiments, the hatching rate and proportional emergence

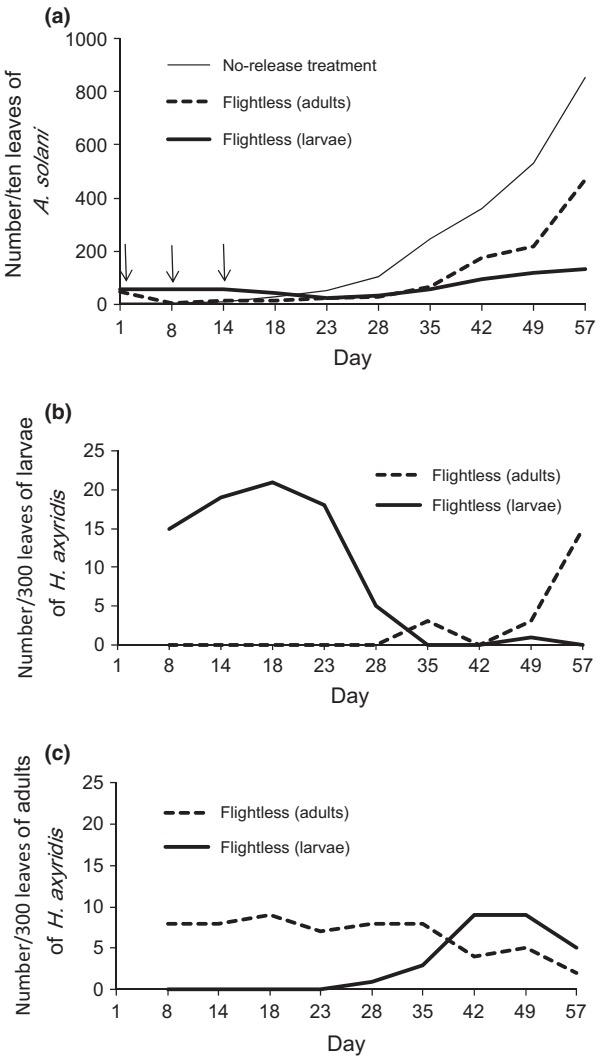

Fig. 2 Variation in time of the number of (a) *Aulacorthum solani* on 10 leaves per plant, (b) larvae of *Harmonia axyridis* and (c) adult *H. axyridis* on 300 leaves of eggplants in small-scale greenhouses. The values of (a) are expressed as medians. Arrows show the day on which second-instar or adult *H. axyridis* were released in each plot. 'Flightless (adults)' means the experimental plot in which adult *H. axyridis* were released. 'Flightless (larvae)' means the experimental plot in which second-instar *H. axyridis* were released. The number of *H. axyridis* larvae that were measured in this release experiment includes all larval stages.

Table 4 Results for generalized linear mixed models examining the effects of variables on the numbers of *Aulacorthum solani* and *Harmonia axyridis* (larvae and adults) in Trial 3

Species	Factor	Coefficient	Wald statistic
A. solani	Treatment (larvae)	0	
	Treatment (adults)	−1.103	−5.65***
	Treatment (no-release)	−0.082	−0.42 ns
	Day	0.023	69.44***
	Treatment × Day (larvae)	0	
	Treatment × Day (adults)	0.038	81.31***
	Treatment × Day (no-release)	0.034	83.97***
Larvae of *H. axyridis*	Treatment (larvae)	0	
	Treatment (adults)	−8.447	−4.89***
	Day	−0.039	−4.95***
	Treatment × Day (larvae)	0	
	Treatment × Day (adults)	0.180	5.45***
Adults of *H. axyridis*	Treatment (larvae)	0	
	Treatment (adults)	3.572	4.37***
	Day	0.071	4.54***
	Treatment × Day (larvae)	0	
	Treatment × Day (adults)	−0.077	−4.36***

The listed variables were selected as the minimum Akaike information criterion model.
***$P < 0.0001$; ns, not significant.

of the flightless strain were lower than those of the wild type (table 2), suggesting that it seems to reflect the inbreeding depression (Seko and Miura 2009; Seko et al. 2012) and/or the correlated response to artificial selection for reduced flight ability, that is, the pleiotropic effects of flightless allele on fitness components (Ueno et al. 2004). A decrease in the viability of the flightless strain may also have caused genetic drift (Hopper et al. 1993; Roush and Hopper 1995; Miyatake 1998; Frankham et al. 2002). The reduced viability is likely to be expected to reduce the effectiveness in controlling aphids. However, the incidence of fourth instars in the flightless plot was on the same or upper level as the wild types (fig. 1b,b'). Two possible explanations for counteracting the lower viability during immature stages are as follows: (i) The residence rate of flightless *H. axyridis* is higher not only during adult stages, but also during immature stages than that of the wild type; and (ii) the flightless females have more opportunity to lay eggs than the wild-type ones because they remain on eggplants longer. The former is likely to be insubstantial because Nakayama et al. (2013) found no differences between the flightless strain and the wild-type strain with regard to residence period on a plant for the first, second and fourth instars of *H. axyridis*. We should compare fecundity between the flightless and the wild-type strains in laboratory experiments incorporating a large spatial scale to verify the latter possibility.

In Trial 1, the wild-type adults appeared earlier than those of the flightless strain (fig. 1c). Although the developmental period of the wild-type strain was shorter for 1 day than that of the flightless strain (table 2), it is not likely to be the primary factor because the peak incidence of flightless adults was

delayed for nearly 3 weeks compared with that of wild-type adults. The following two factors may explain the time difference. First, there were differences in aphid density between treatments. In the flightless plot of Trial 1, the proportion emergence of *H. axyridis* larvae, which was derived from the first and second release, might be lower than that of wild-type plot because the aphid density from days 1 to 22 was too low to remain on eggplants longer and complete the development for *H. axyridis* larvae. By contrast, in the flightless plot of Trial 2, *H. axyridis* adults appeared earlier for 10 days compared with the wild-type strain (fig. 1c′). The aphid density was 5.5–9.0 and 19.0–40.5 individuals on six leaves per plant in the wild-type plot and the flightless plot, respectively, when the fourth and fifth release of *H. axyridis* larvae was performed. Second, the population of *H. axyridis* adults might include the offspring of released *H. axyridis*. In the flightless plot of Trial 1, the increase in *H. axyridis* adults from days 64 to 81 might reflect the emergence of the offspring of released *H. axyridis*. There is a possibility that this factor masks the net residence period of released *H. axyridis* on plants. We could not distinguish *H. axyridis* that were released from the offspring due to the difficulty in marking on *H. axyridis* larvae.

In Trial 3, both treatments resulted in lower numbers of *A. solani* than in untreated controls (fig. 2a). Our results suggest that the release of both two adults and ten second instars of the flightless strain is effective in controlling *A. solani* at an initial aphid density of nearly 50 individuals per ten leaves of eggplants. The number of *A. solani* was suppressed, while 7–9 *H. axyridis* adults on 300 leaves of eggplants were observed on the plants from days 8 to 35 in the treatment in which adults were released, suggesting that the density of *H. axyridis* adults can be effective in controlling *A. solani* on eggplants. On the other hand, GLMMs revealed significant treatment and treatment × day interaction effects on the number of *A. solani* (table 4), suggesting that the incidence varied depending on the day between the release plots of larvae and adults. Actually, the number of *A. solani* tended to be higher in the treatment in which larvae were released than in those in which the adults were released until day 23, but it was reversed after day 35 (fig. 2a). In the treatment in which second instars were released, some *H. axyridis* adults emerged on day 28, which corresponded to a disappearance of larvae (fig. 2c). This result suggests that the release of larvae was effective in suppressing *A. solani* during immature stages and after emergence. By contrast, in the treatment in which adults were released, the number of *A. solani* increased as the offspring of released adults was observed (fig. 2a,b). The following two factors may explain the short duration of effective control compared with the release of larvae. First, number of *H. axyridis* adult decreased drastically after day 35 in the treatment in which adults were released (fig. 2c). The reduction could lead to the increase in *A. solani* from day 35 onward. They might die a natural death because the longevity of female adults of flightless *H. axyridis* after emergence was about 55 and 30 days at 20 and 25°C, respectively (T. Seko and K. Miura unpublished). Second, the incidence of *H. axyridis* larvae could be delayed because of the shortage of prey and the presence of conspecific adults. Field and laboratory experiments indicate that ladybird beetles tend to lay eggs where there are sufficient numbers of aphids to ensure the survival of the first instars (Dixon 1959; Wratten 1973; Seagraves 2009). The initial density of *A. solani* before our release experiment was estimated to be the lowest that would enable capture of enough aphids for larvae to develop, but not enough for females to lay eggs. However, the aphid densities required for ovariole maturation are presumably equal to the minimum densities needed for survival of coccinellid larvae (Dixon 1970; Honek 1980). On the other hand, coccinellids avoid oviposition when they come in contact with predators or evidence of their presence to avoid intraguild predation and cannibalism (Hemptinne et al. 1992; Merlin et al. 1996; Yasuda et al. 2000; Mishra and Omkar 2006). It is possible that, in the treatment in which flightless adults were released, the flightless adults did not produce offspring until the number of conspecific adults decreased. From these points of view, the larval release of flightless strain may be beneficial to the duration of effective control compared with the release of adults.

From our research findings, we conclude that it is more effective to release larvae of flightless *H. axyridis* than wild-type larvae or flightless adults. The lower viability of the flightless strain may provide a rise in the cost of mass rearing compared with the wild type. However, the effects of inbreeding would be avoided by introducing a hybridization programme because viability of a hybrid line was greater than that of two isofemale lines in flightless *H. axyridis* (Seko et al. 2012). Although the flightless strain, which was used in our study, was not same as isofemale lines, which were used in Seko et al. (2012), the hybrid vigour should be effective to recover its viability if the reduction is due to inbreeding depression. The release of larvae could be more cost–effective than that of adults. In our study, second instars exhibited a higher

ability to suppress *A. solani* compared with adults; nevertheless, the production cost of 10 s instars corresponded to a half of two adults. Economic injury levels and economic thresholds have been estimated for *A. solani* on pepper (Sanches et al. 2007) and for *A. gossypii* on citrus (de Mendoza et al. 2001), but not for aphid species on eggplant. We need the information on eggplant to verify the effectiveness of aphid control and the potential economic benefit using larvae of flightless *H. axyridis* precisely. Although our findings are preliminary and research can be further improved, it provides the first implication of the cost–effectiveness in controlling aphids using larvae of flightless ladybirds. Even though the status of *H. axyridis* in biological control is doubtful in Europe and America because this species is recognized as invasive alien species (Koch and Galvan 2008; van Lenteren et al. 2008; Brown et al. 2011), the release stage also can be an important factor influencing its effectiveness in controlling aphids and has potential economic benefits for other flightless ladybird beetles such as *A. bipunctata*.

Acknowledgements

We thank Agrisect Inc. for providing the ladybird beetles. We also thank Dr. K. Tabuchi, from the NARO Tohoku Agricultural Research Center, for his valuable advice on statistical analysis. This research was supported by research and development projects for application in promoting new policy by Agriculture Forestry and Fisheries from the Ministry of Agriculture Forestry and Fisheries of Japan. We also thank Dr. S. Lommen, from University of Fribourg, and an anonymous reviewer for providing helpful comments on an earlier version of the manuscript.

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